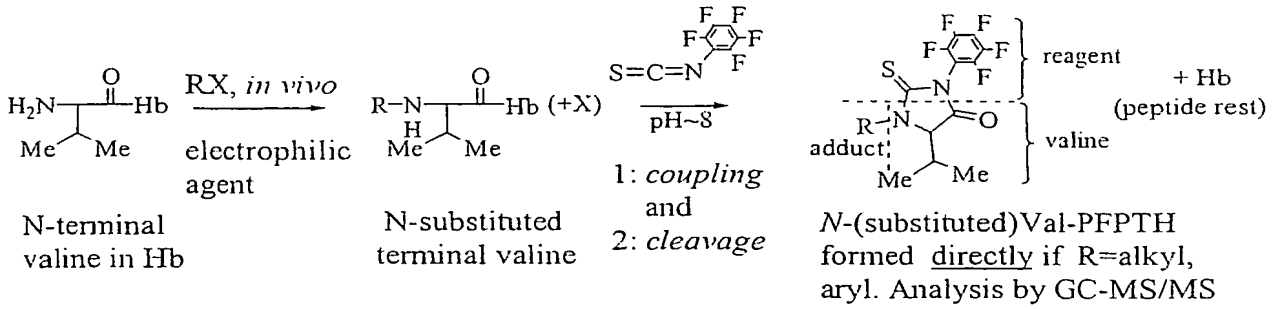
Figure 1. Principles of the N-alkyl Edman procedure[a].



N-terminal valine in Hb

N-substituted terminal valine

N-(substituted)Val-PFPTH formed directly if R=alkyl, aryl. Analysis by GC-MS/MS

Footnote: [a]Phenyl isothiocyanate and pentafluorophenyl isothiocyanate have been utilized in this procedure.

Figure 2. Principles of the present method of the first aspect

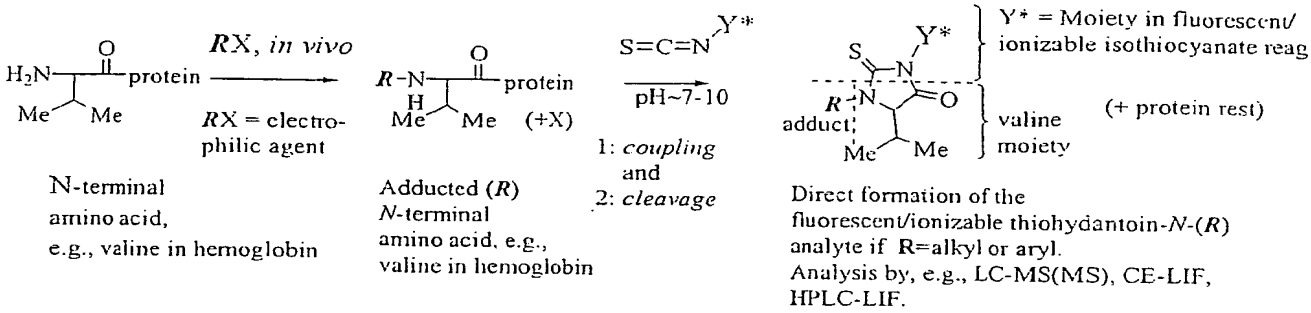


Figure 3. The reactions and reagents studied: coupling between selected isothiocyanate reagents (**Y**-N=C=S) and valine itself (***R***=H, **X**=O⁻) or bound (***R***= alkyls) to a peptide (**X**=LeuSer) or protein (**X**=rest of human globin).

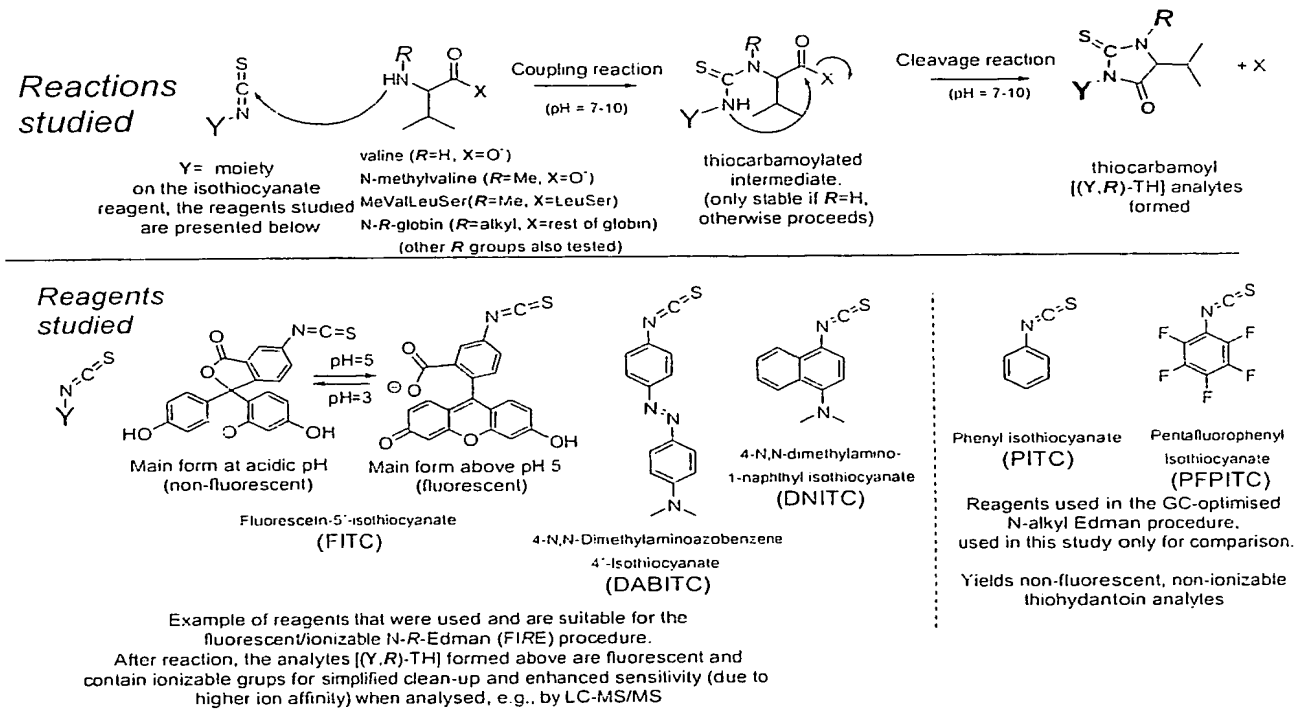
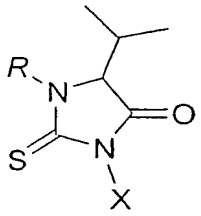
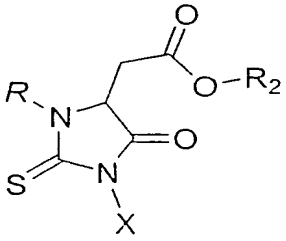


Figure 4. General structure of the adducted analytes and proposed adducted analytes formed in the present method from N-terminal valine, e.g., in proteins such as globin (XTH-*R*-Val)[a] and of analytes formed from N-terminal asparagine, e.g., in proteins such as bovine serum albumin (XTH-R-Asp-R_2)[b].



XTH-*R*-Val

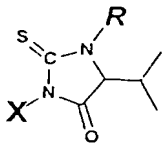


XTH-*R*-Asp-R_2

Footnotes: [a]The *R* substituent in the valine thiohydantoin represents any adduct (e.g., alkyl and aryl or substituted homologues of alkyl and aryl, but not hydrogen) covalently bound to the valine nitrogen. The X substituent in the valine thiohydantoin represents the moiety of any isothiocyanate reagent utilized in which the isothiocyanate group is directly bound to an aromatic ring or an aromatic ring system, thereby providing fluorescent and/or ionizable and/or other valuable properties to the analyte. However, X is not a phenyl, 4-bromophenyl, 4-methoxyphenyl or pentafluorophenyl group, e.g., PITC and PFPITC.

The *R* substituent in the asparagine thiohydantoin represents any adduct (e.g., alkyl and aryl or substituted homologues of alkyl and aryl, but not hydrogen) covalently bound to the valine nitrogen. The X substituent in the asparagine thiohydantoin represents the moiety of any isothiocyanate reagent utilized in which the isothiocyanate group is directly bound to an aromatic ring or an aromatic ring system, thereby providing fluorescent and/or ionizable and/or other valuable properties to the analyte. However, X is not a phenyl, 4-bromophenyl, 4-methoxyphenyl or pentafluorophenyl group, e.g., PITC and PFPITC. The R_2 substituent on the carboxyl group of asparagine represents hydrogen; an alkyl, aryl, carboxyl or benzyl group; or substituted analogues of these. This carboxyl group can also be an anion.

Figure 5. Studied thiohydantoin analytes.



Analyte	(nr)	Adduct (*R*)	Reagent used (X)
PTH-Val	1	R=H	PITC
PTH-MeVal	2	R=Me	PITC
PFPTH-MeVal	3	R=Me	PFPITC
PFPTH-HOEtVal	4	R=CH_2CH_2OH	PFPITC
DABTH-Val	5	R=H	DABITC
DABTH-MeVal	6	R=Me	DABITC
DNTH-Val	7	R=H	DNITC
DNTH-MeVal	8	R=Me	DNITC
FTH-Val	9	R=H	FITC
FTH-MeVal	10	R=Me	FITC
FTH-AAVal	11	R= $CH_2CH_2CONH_2$	FITC
FTH-GAVal	12	R=$CH_2CH(OH)CONH_2$	FITC
FTH-HOC_{18}Val	13	R=$CH_2CH(OH)(CH_2)_{15}CH_3$	FITC
FTH-HOPrVal	14	R=$CH_2CH(OH)CH_2$	FITC
FTH-CholEOVal (adduct from cholesterol-5α,6α-epoxide)	15	R=$C_{27}H_{47}O$	FITC
FTH-GlcVal (adduct formed after reduction of glycosylated N-terminal) valine in peptide and globin	16	R=$CH_2[CH(OH)]_4CH_2OH$	FITC

Figure 6. Separation of FTH-Val (**9**) and FTH-MeVal (**10**) by capillary electrophoresis employing detection with a diode array. FTH-Val (**9**) elutes after 7.44 min and FTH MeVal (**10**) elutes after 8.91 min (17 mM phosphate buffer, pH 7, containing 20 mM SDS). Conditions 30 kV, 52 cm capillary, 1 nL sample injected.

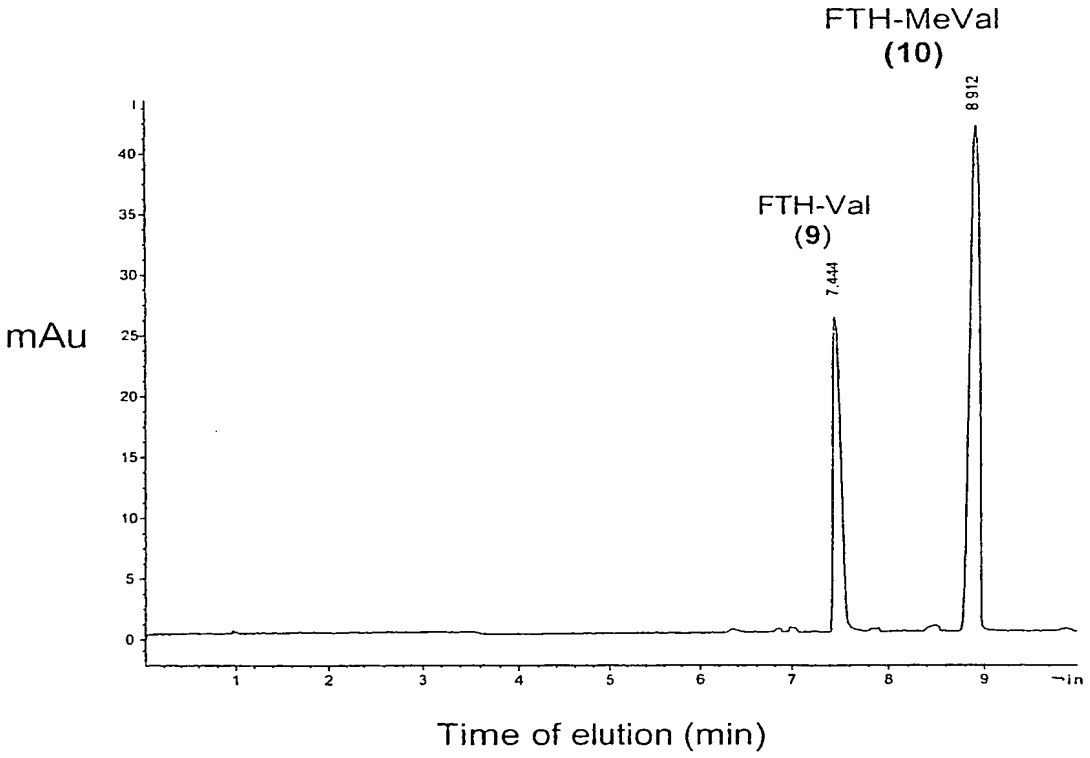
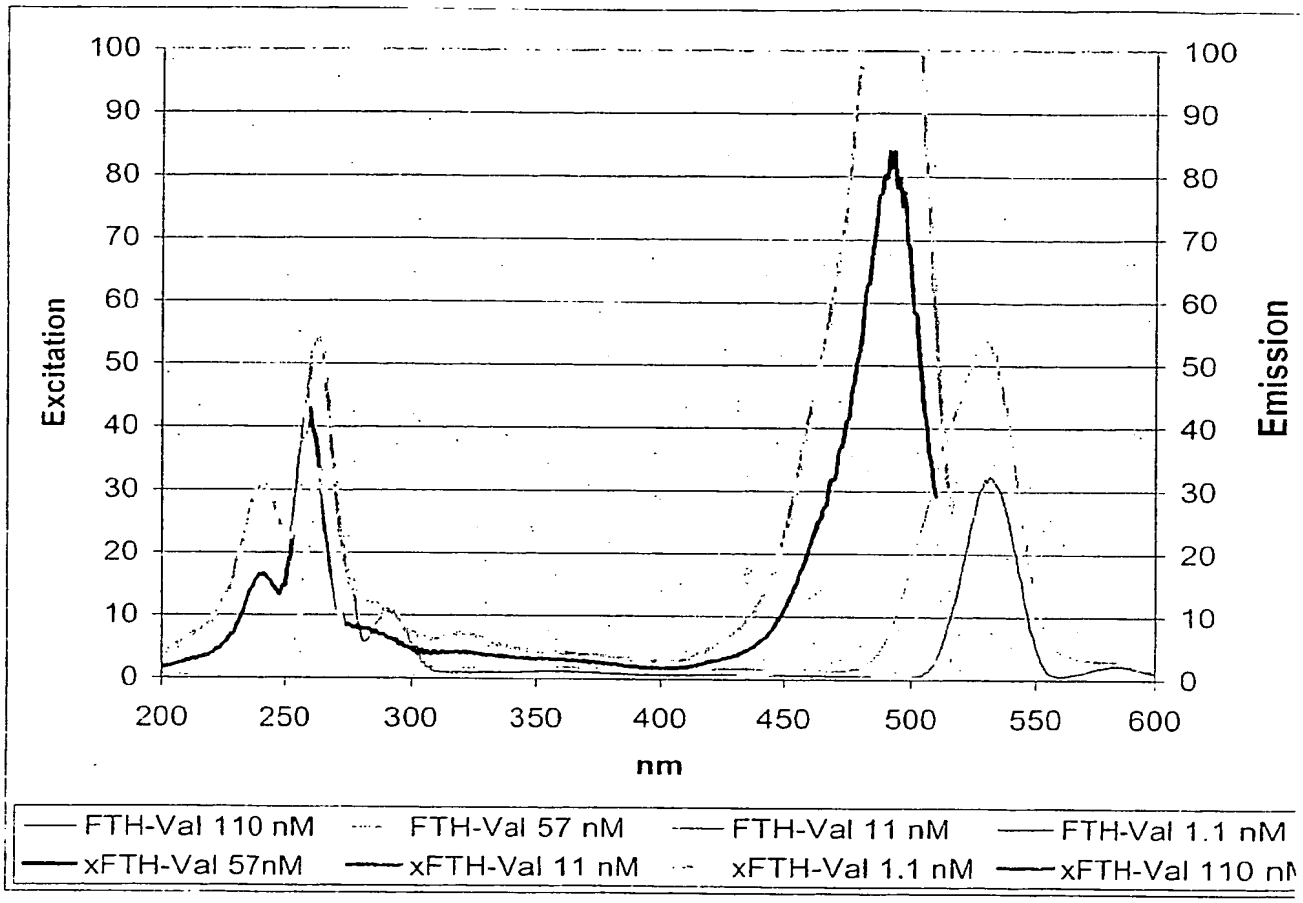
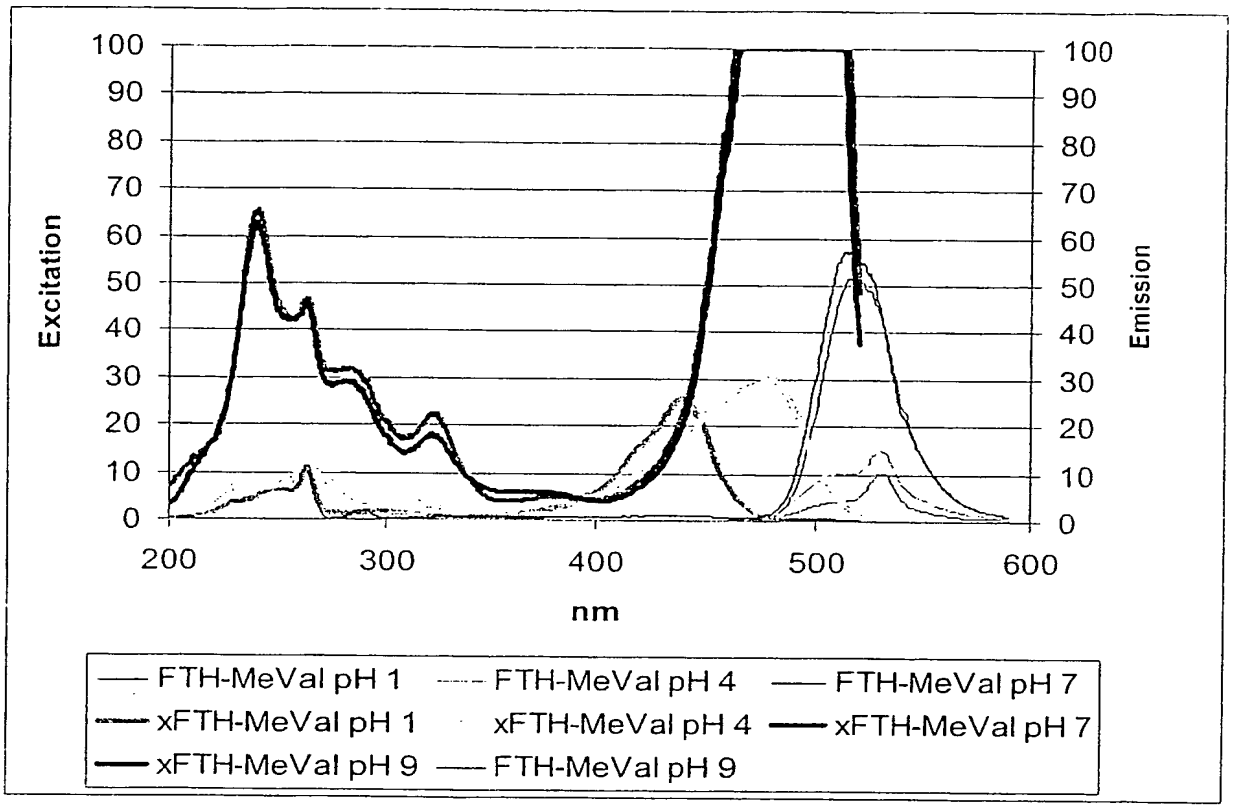


Figure 7. Fluorescence measurements[a]: the excitation and emission spectra of FTH-Val (**9**) at various concentrations.



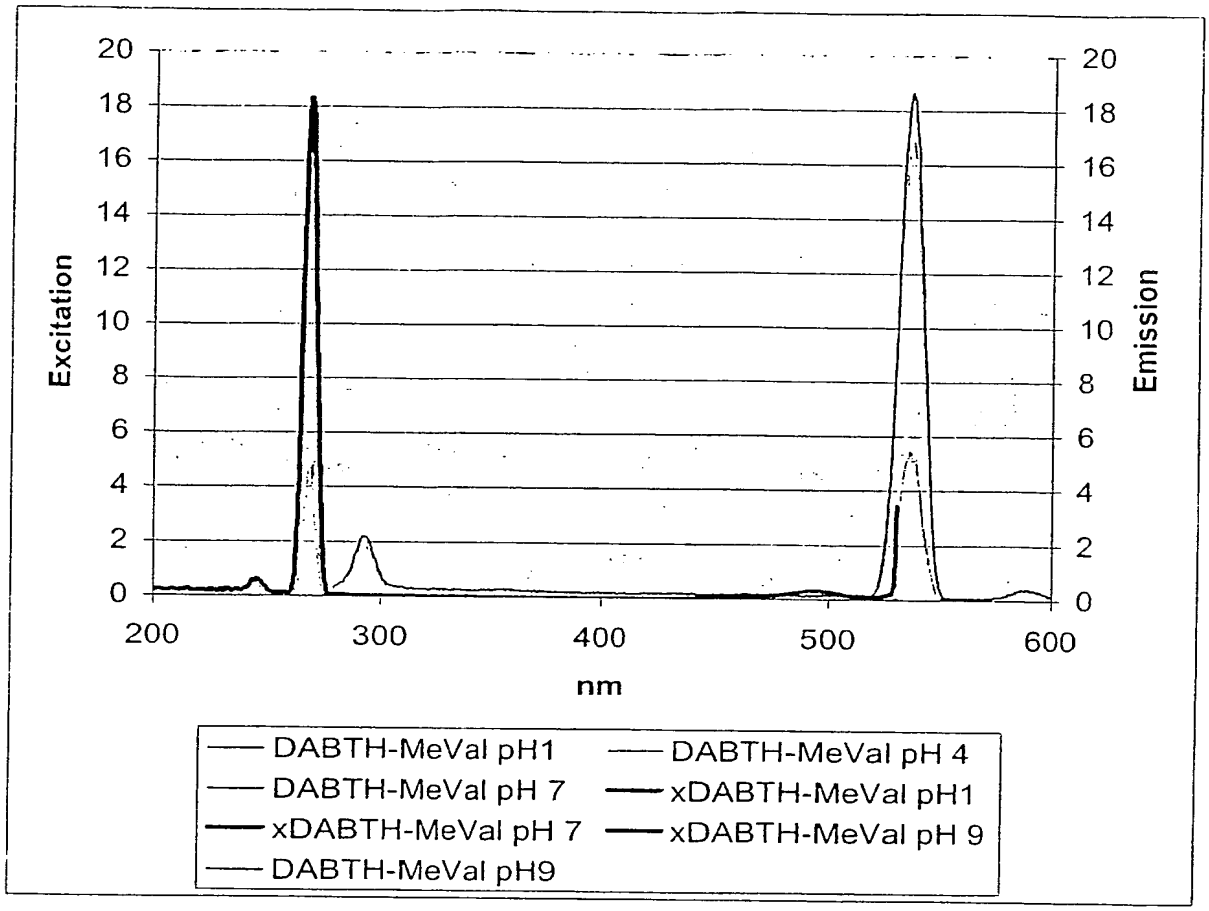
Footnote: [a] FTH-Val (**9**) dissolved in acetonitrile:aqueous buffer (1:9), pH 7, at different concentrations. Excitation wavelength 265nm, emission scan 275-600nm; and emission wavelength 517 nm, excitation scan 200-510nm. The bold lines (xFTH-Val) represent the excitation spectra, while the thin lines (FTH-Val) depict the emission spectra.

Figure 8. Fluorescence measurements[a]: the excitation and emission spectra of 1.1 µM FTH-MeVal (**10**) at pH 1, 4, 7 and 9.



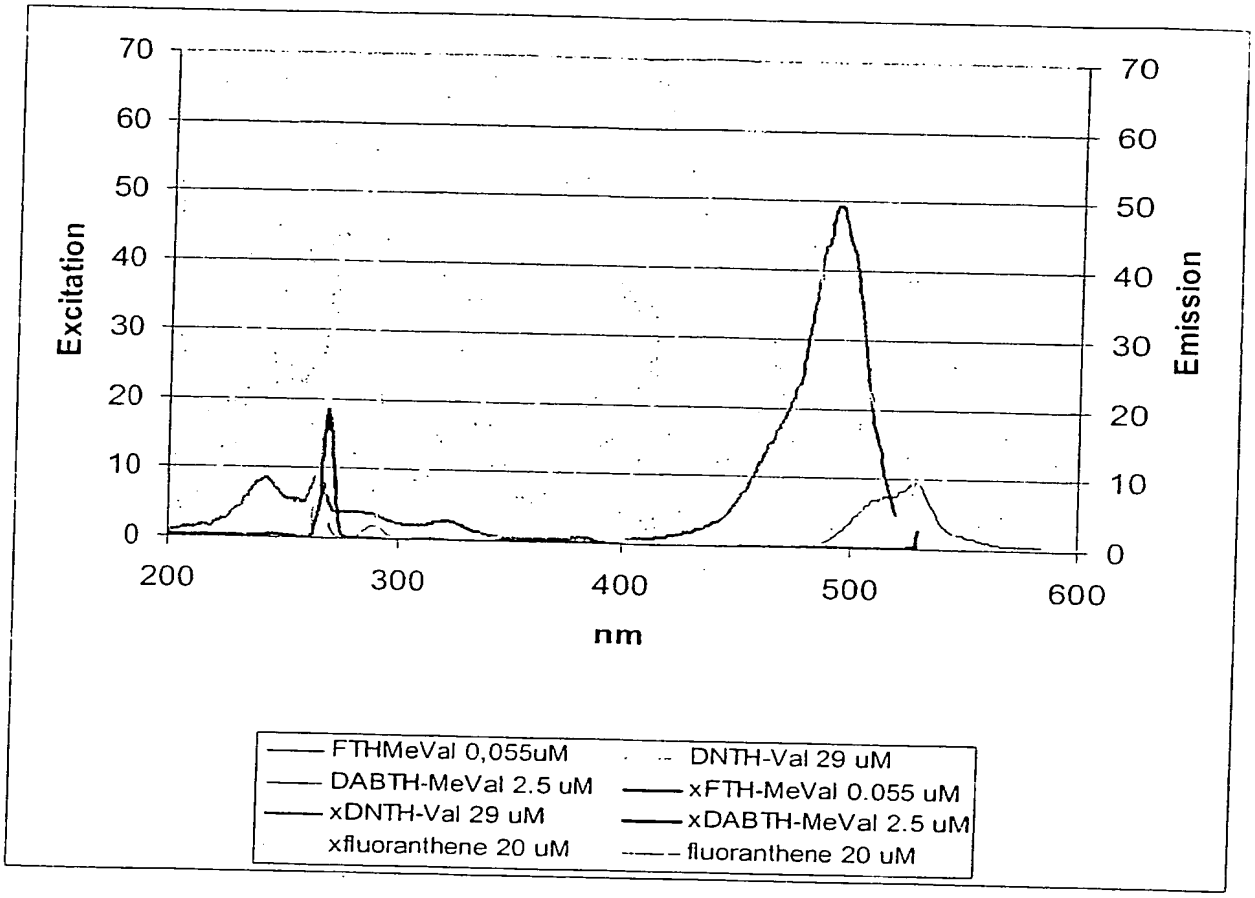
Footnote: [a]The analytes were dissolved in acetonitrile:aqueous buffer (1:9). Slits 5/5; excitation wavelength 265 nm, emission scan 275-600 nm; and emission wavelength at 530 nm, excitation scan 200-520 nm. The bold lines (xFTH-MeVal at pH 1, 4, 7 and 9) represent the excitation spectra, while the thin lines (FTH-MeVal pH 1, 4, 7 and 9) depict the emission spectra.

Figure 9. Fluorescence measurements[a]: the excitation and emission spectra of 2.5 µM DABTH-MeVal at pH 1, 4, 7 and 9.



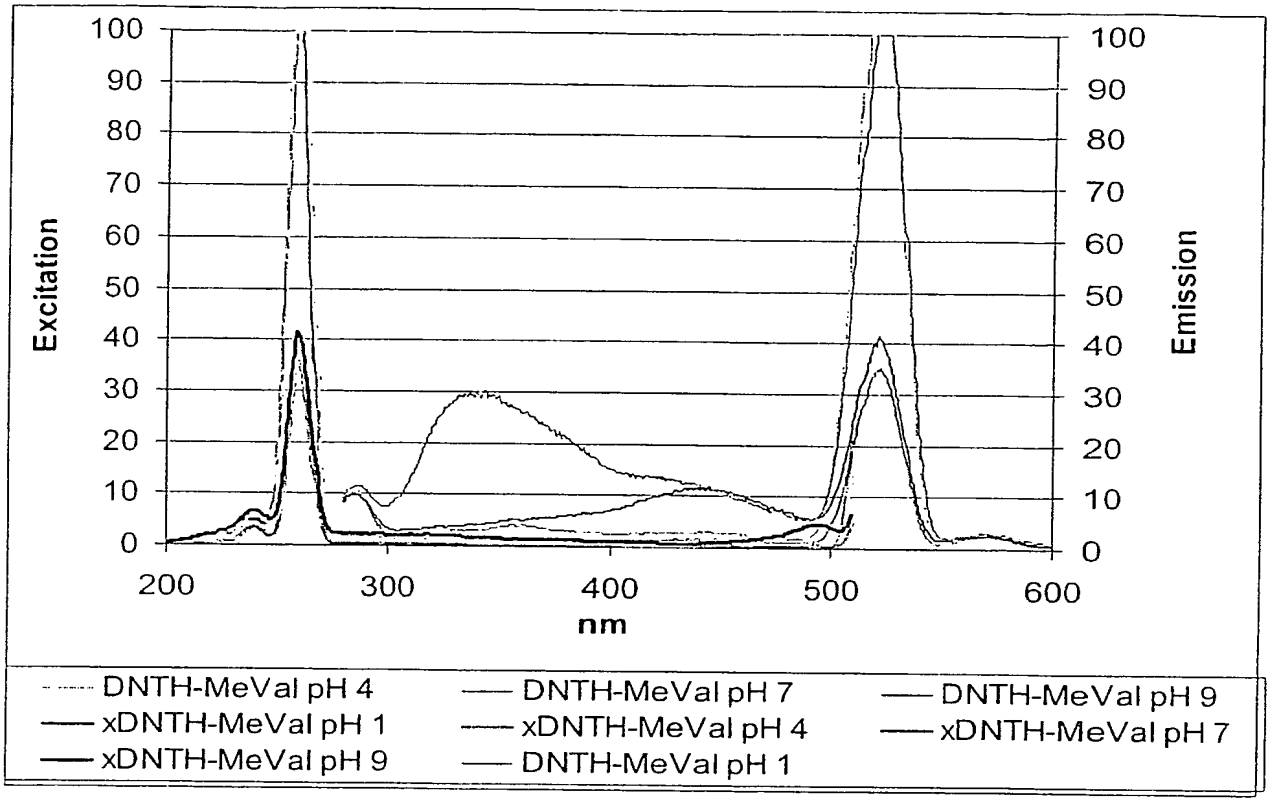
Footnote: [a]The analytes dissolved in acetonitrile:aqueous buffer (1:9). Slits 5/5; excitation wavelength 268 nm, emission scan 280-600 nm; and emission wavelength 538 nm, excitation scan 200-530 nm. The bold lines (xDABTH-MeVal) represent the excitation spectra, while the thin lines (DABTH-MeVal pH) depict the emission spectra.

Figure 10. Comparative studies[a] of the excitation and fluorescence emission spectra for the analytes DABTH-MeVal (**6**), DNTH-Val (**7**), FTH-MeVal (**10**), using fluoranthene as a reference.



Footnote: [a]The compounds were dissolved in acetonitrile:aqueous buffer (1:9) at a pH suitable for each analyte. Slits 5/5 and locked excitation/emission wavelength suitable for each analyte. The bold lines represent the excitation spectra, while the thin lines depict the emission spectra.

Figure 11. Fluorescence measurements[a]: the excitation and emission spectra of 1.5 µM DNTH-MeVal at pH 1, 4, 7 and 9.



Footnote: [a]The compounds were dissolved in acetonitrile:aqueous buffer (1:9). Slits 10/10; excitation wavelength 260 nm; emission scan 270-600 nm and emission wavelength 520 nm, excitation scan 200-510 nm. The bold lines (xDNTH-MeVal) represent the excitation spectra, while the thin lines (DNTH-MeVal) depict the emission spectra.

Figure 12. UV absorbance of selected analytes in acetonitrile, using fluoranthene as reference.

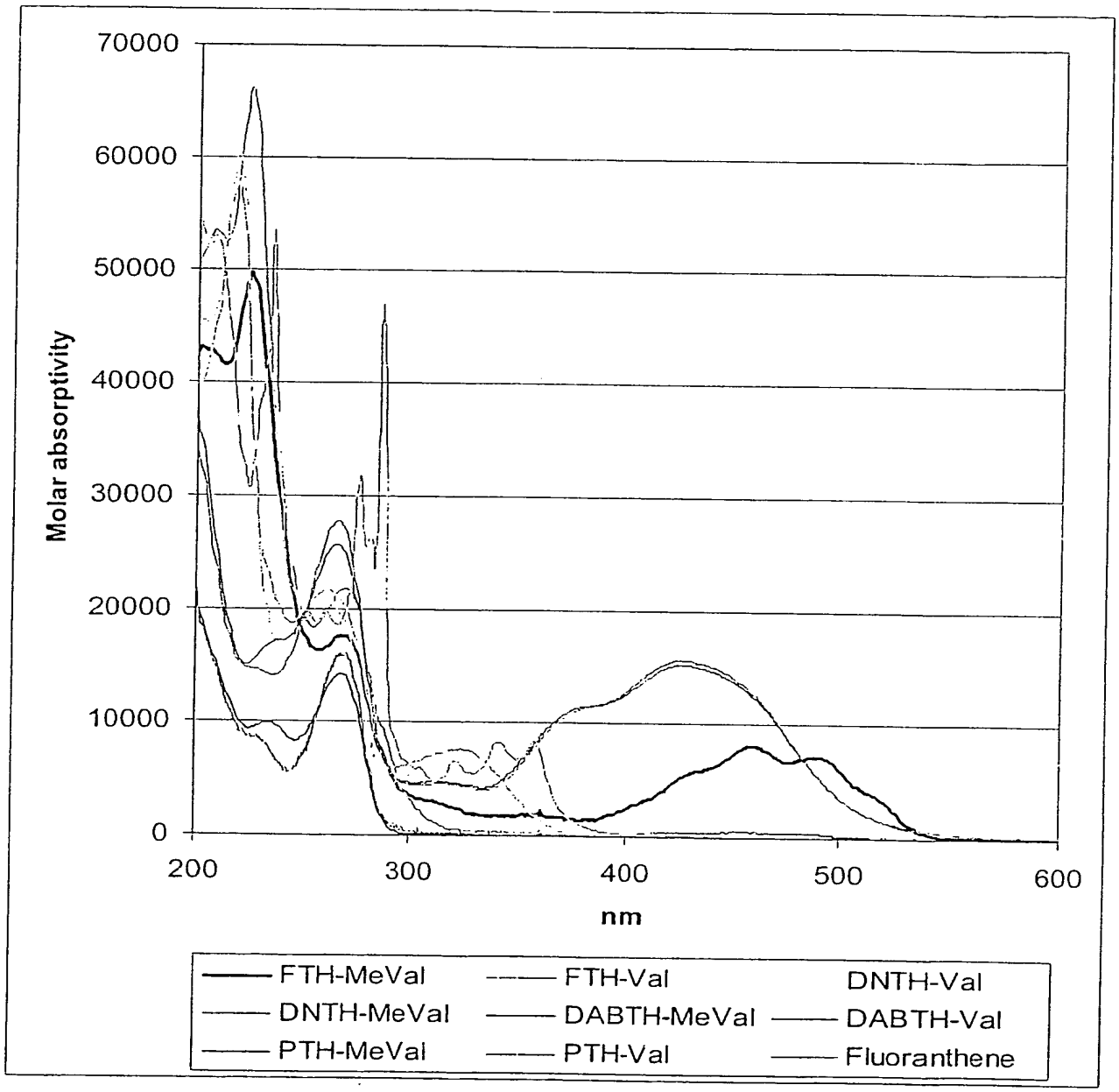


Figure 13. UV absorbance for selected analytes dissolved in acetonitrile/water (1/1) containing 0.1 % TFA, using fluoranthene as reference.

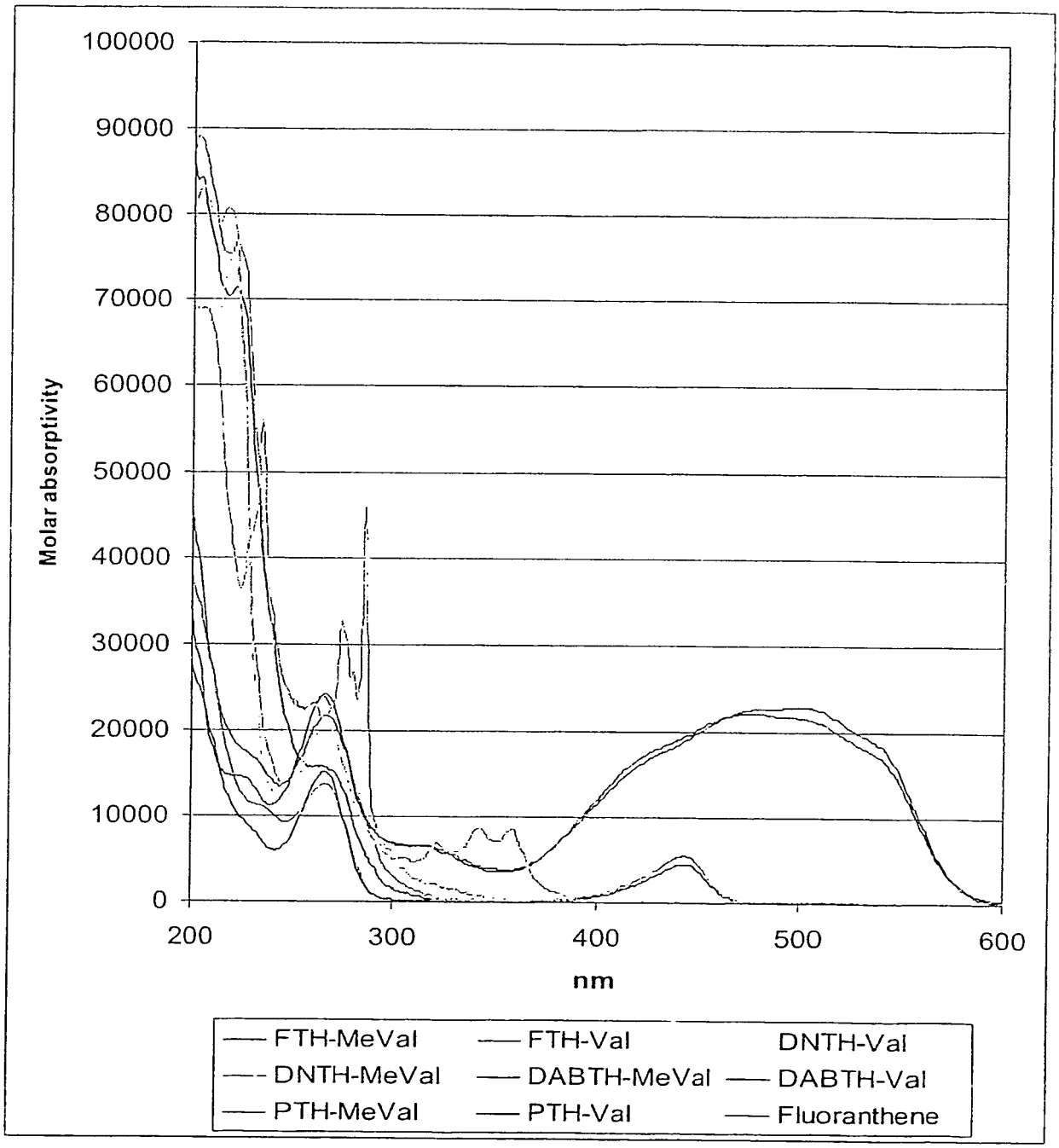


Figure 14. LC-MS/MS analysis of acrylamide adducts of human globin using the present method, in comparison with reference compounds.

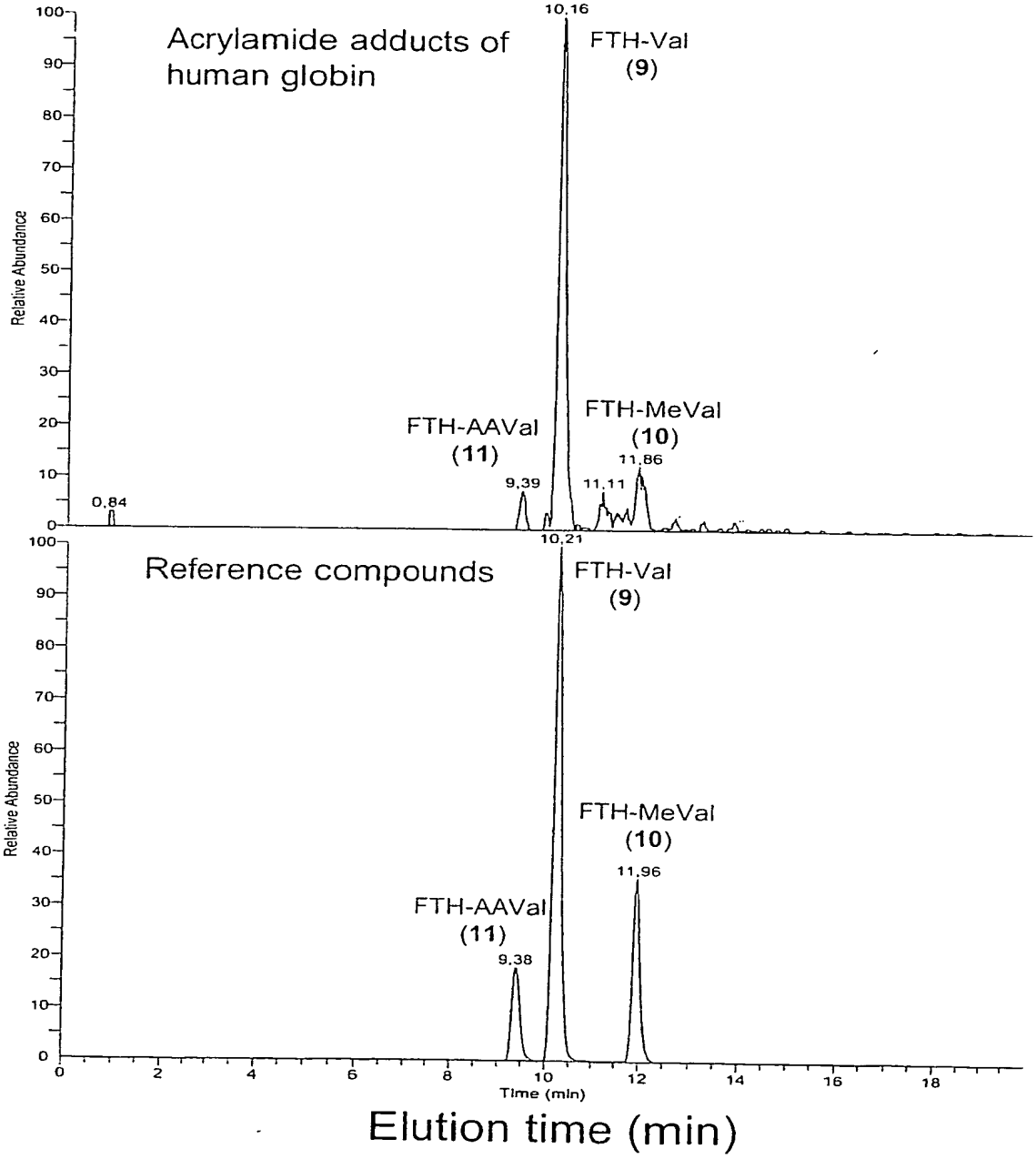
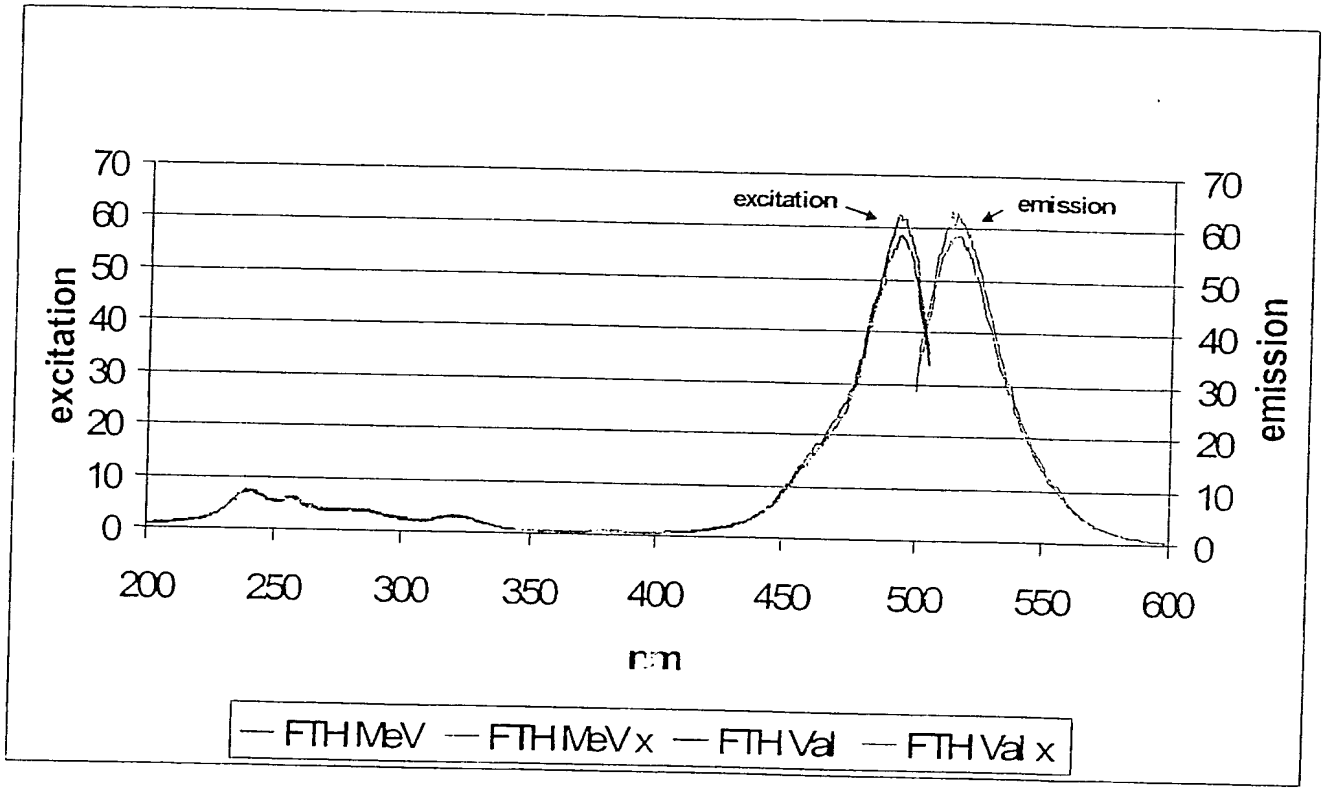


Figure 15. Fluorescence measurements[a]; the excitation and emission spectra of 0.1μM FTH-Val (**9**) and FTH-MeVal (**10**) at pH 7.



Footnote: [a]The analytes were dissolved in acetonitrile:aqueous buffer (1:9). Slits 5/5; excitation wavelength 492 nm, emission scan 500-600 nm; and emission wavelength 515 nm, excitation scan 200-505 nm. The bold lines represent the excitation spectra; while the thin lines (x) depict the emission spectra.

Figure 16. LC-MS/MS analysis of: FTH-GlcVal (a) formed from a glycosylated, reduced model dipeptide and of FTH-GlcVal (b) formed from glycosylated reduced globin.

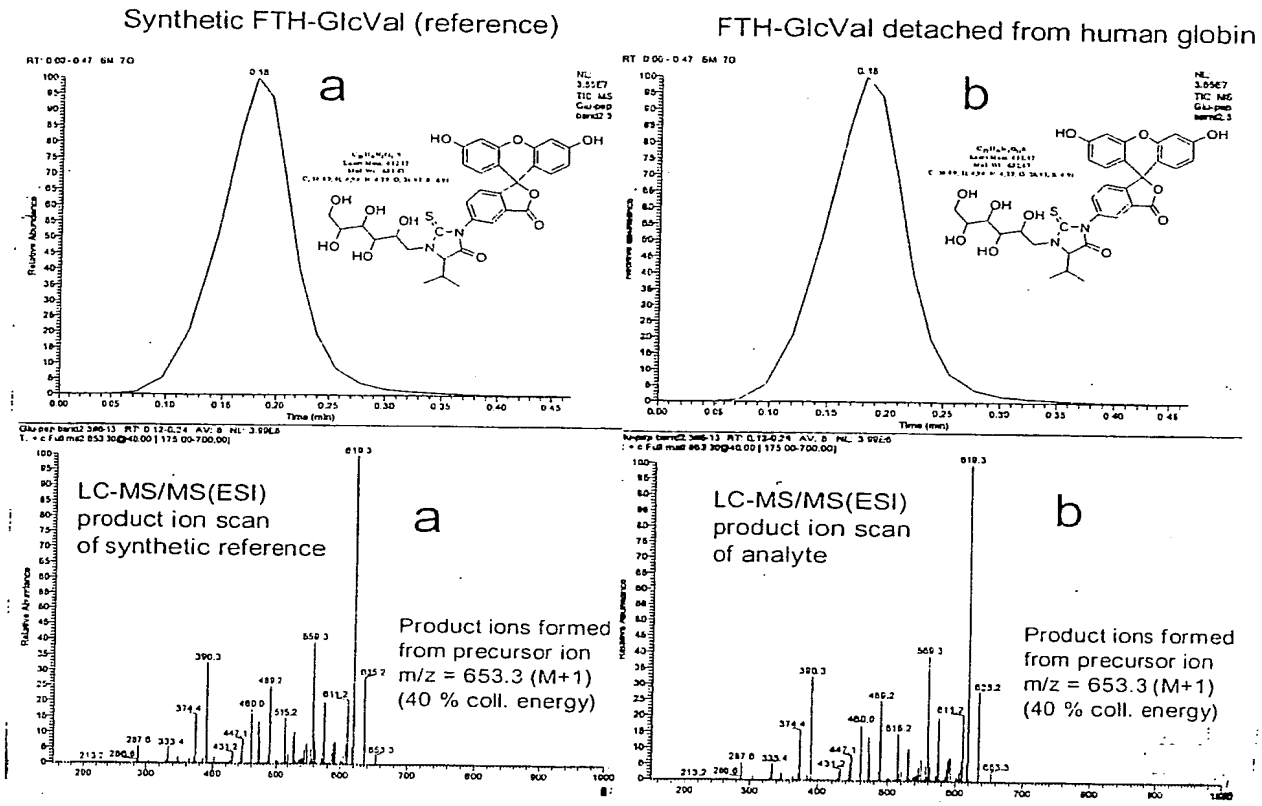


Figure 17. Comparison of the relative sensitivities (presented on a log scale) obtained by determination of the limits of detection (LOD) of LC-MS/MS in the ESI and APCI modes.

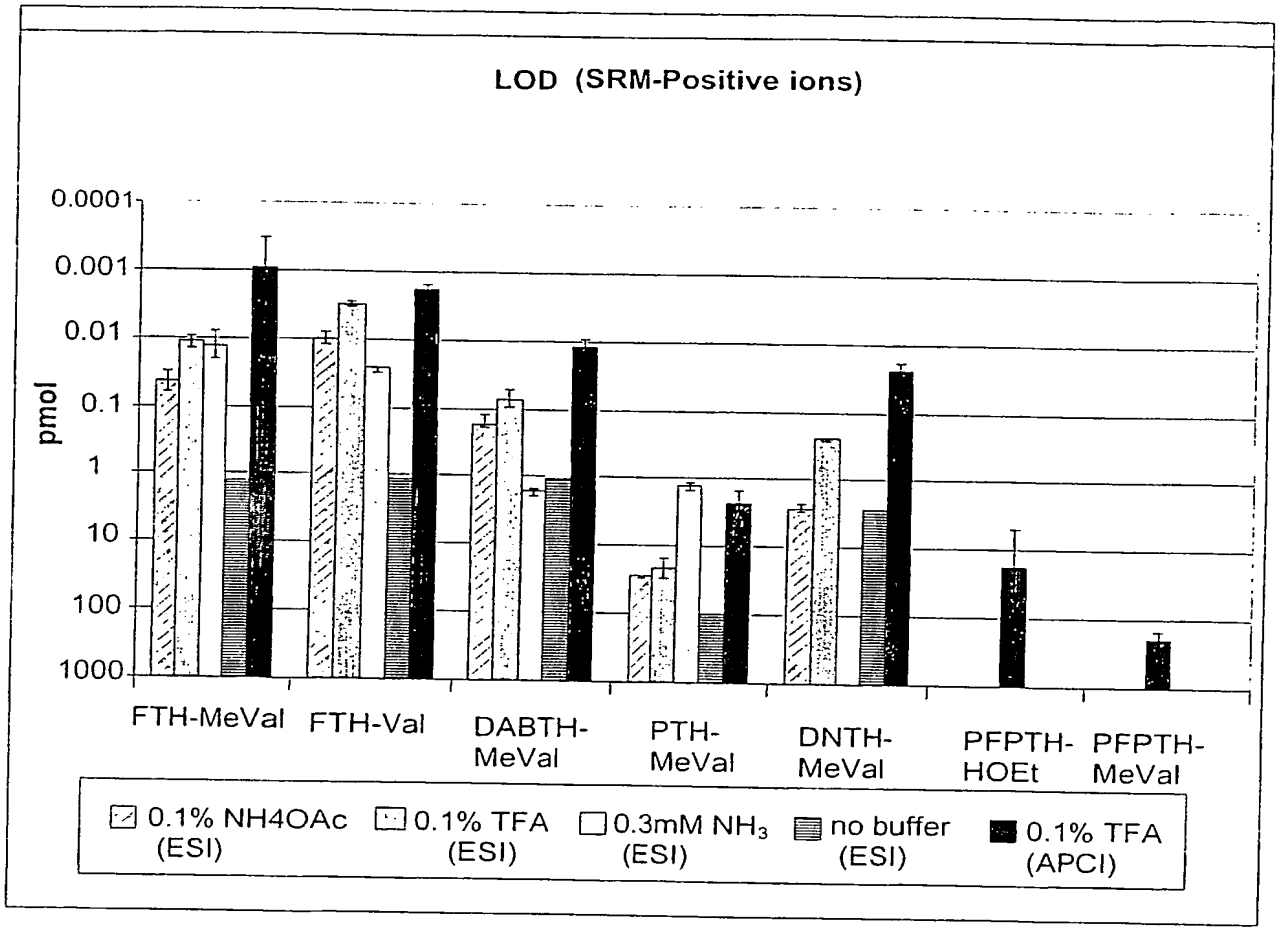


Figure 18. Comparison of the relative sensitivities (presented on a log scale) obtained by determinations of the limits of detection (LOD) of LC-MS/MS in the ESI and APCI modes.

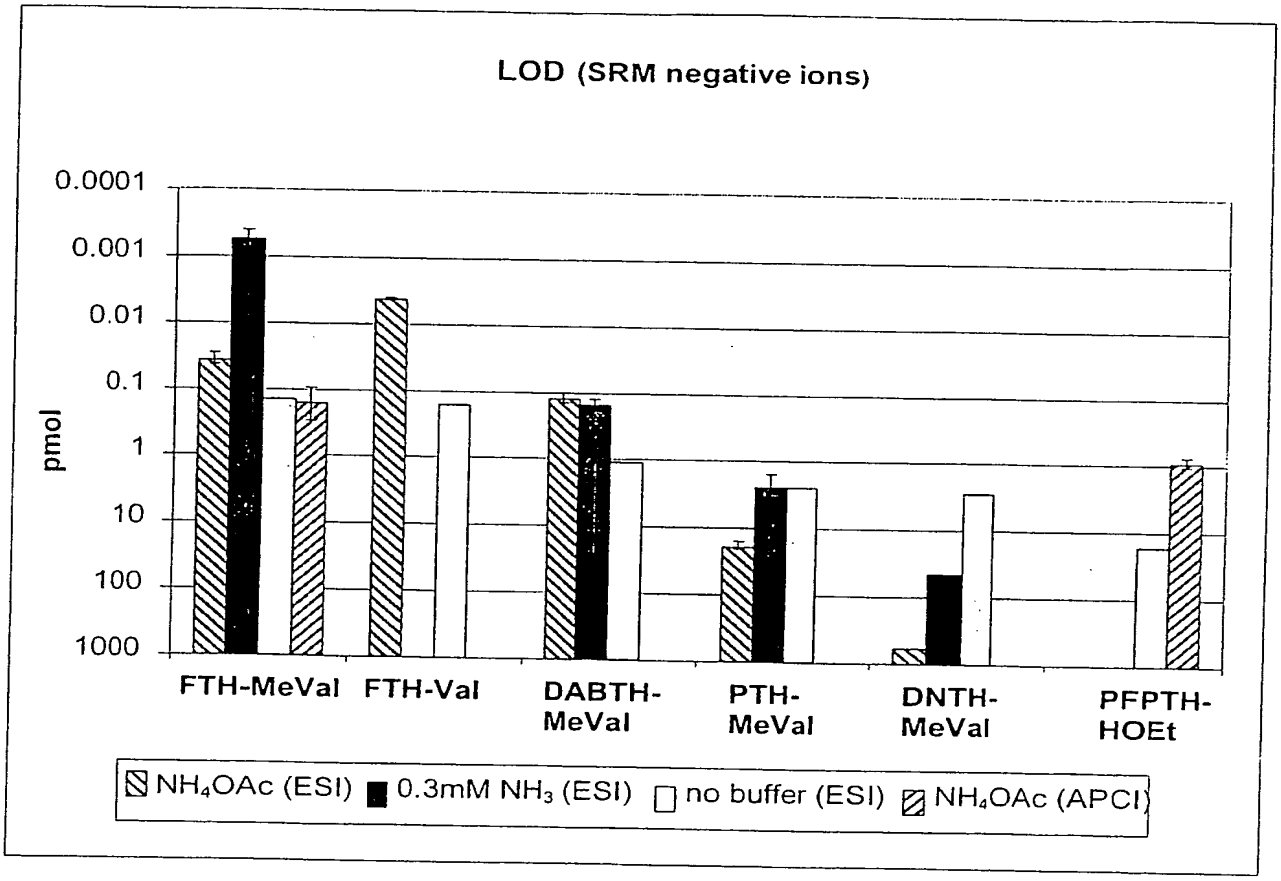


Figure 19. Illustration of a simplified approach to sample preparation and clean-upbased on the principles of the fluorescent/ionizable N-R-Edman procedure.